Nonenzymatic Glycosylation In Vitro and in Bovine Endothelial Cells Alters Basic Fibroblast Growth Factor Activity

A Model for Intracellular Glycosylation in Diabetes

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Abstract

Intracellular sugars are more reactive glycosylating agents than glucose. *In vitro* nonenzymatic glycosylation of basic fibroblast growth factor (bFGF) by fructose, glucose-6-phosphate (G6P), or glyceraldehyde-3-phosphate (G3P) reduced high affinity heparin-binding activity of recombinant bFGF by 73, 77, and 89%, respectively. Mitogenic activity was reduced 40, 50, and 90%. To investigate the effects of bFGF glycosylation in GM7373 endothelial cells, we first demonstrated that GLUT-1 transporters were not downregulated by increased glucose concentration. In 30 mM glucose, the rate of glucose transport increased 11.6-fold, and the intracellular glucose concentration increased sixfold at 24 h and fivefold at 168 h. The level of total cytosolic protein modified by advanced glycosylation endproducts (AGEs) was increased 13.8-fold at 168 h. Under these conditions, mitogenic activity of endothelial cell cytosol was reduced 70%. Anti-bFGF antibody completely neutralized the mitogenic activity at both 5 and 30 mM glucose, demonstrating that all the mitogenic activity was due to bFGF. Immunoblotting and ELISA showed that 30 mM glucose did not decrease detectable bFGF protein, suggesting that the marked decrease in bFGF mitogenic activity resulted from posttranslational modification of bFGF induced by elevated glucose concentration. Cytosolic AGE-bFGF was increased 6.1-fold at 168 h. These data are consistent with the hypothesis that nonenzymatic glycosylation of intracellular protein alters vascular cell function. (*J. Clin. Invest.* 94:110–117.) Key words: glucose • advanced glycosylation end products • heparin • mitogens • cytokines

Introduction

Chronic hyperglycemia appears to be the central initiating factor responsible for the development of diabetes-specific vascular and neurologic damage (1). Hyperglycemia-induced biochemical abnormalities implicated in this process include increased polyol pathway activity and associated alterations in the redox state of pyridine nucleotides (2–4), increased synthesis of diacylglycerol with the consequent activation of several isoforms of protein kinase C (5–9), decreased myoinositol-dependent Na/K-ATPase activity (10–13), and covalent modification of proteins by nonenzymatic glycosylation (14–18).

Nonenzymatic glycosylation has been shown to cause qualitative and quantitative changes in extracellular matrix components (19–23) which can affect cell adhesion, growth, and matrix production (24–30). Many of these effects are mediated by advanced glycosylation endproducts, which form from 1-amino-1-deoxyketose adducts through a complex series of dehydrations, rearrangements, and redox reactions (31).

Although hemoglobin was the first protein for which nonenzymatic glycosylation was demonstrated to reflect time-integrated glucose concentration (32–35) and liver alcohol dehydrogenase has also been shown to be glycosylated in vivo (36), most recent work has focused on long-lived extracellular macromolecules, since the rate of nonenzymatic glycosylation is a function of both sugar concentration and time.

Glucose has the slowest rate of glycosylation product formation of any naturally occurring sugar, however, because the rate of Schiff base formation is directly proportional to the percentage of sugar in the open chain form (37). Thus the rate for such intracellular sugars as fructose, glucose-6-phosphate and glyceraldehyde-3-phosphate is considerably faster than the rate for glucose (38). The elevated levels of these compounds that can occur during hyperglycemia (39, 40) would therefore modify intracellular proteins at a much more rapid rate than glucose.

For this reason, we hypothesized that increased nonenzymatic glycosylation of intracellular proteins in endothelial cells exposed to diabetic levels of hyperglycemia would result in altered function of proteins that regulate endothelial cell growth. Basic fibroblast growth factor (bFGF)[1] was selected as a model for study, because none of its isoforms contain a signal peptide sequence. Thus, bFGF is not secreted, but remains stored intracellularly until it is released by endothelial injury (41). In this study, we have evaluated the effect of in vitro nonenzymatic glycosylation on bFGF heparin-binding activity and its mitogenic activity; characterized the effect of high extracellular glucose concentration on GM7373 endothelial cell glucose transporter expression, rate of glucose transport, intracellular glucose concentration and level of nonenzymatically glycosylated cytosolic protein; and then measured the effect of high extracellular glucose concentration and its intracellular consequences on GM7373 endothelial cell cytosol bFGF-mediated mitogenic activity.

Methods

Iodination of bFGF. Human recombinant bFGF (R&D Systems Inc., Minneapolis, MN) was labeled by Enzymobeads (BioRad, Laboratories,

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1. *Abbreviations used in this paper:* AGE, advanced glycosylation endproduct; bFGF, basic fibroblast growth factor; ECGF, endothelial cell growth factor; G3P, glyceraldehyde-3-phosphate; glucose-6-phosphate.

Richmond, CA) with Na ^{125}I (17 Ci/mg; New England Nuclear, Boston, MA) according to established protocols (42).

Nonenzymatic glycosylation of bFGF. bFGF was glycosylated by fructose, G6P, or G3P (Sigma Chemical Co., St. Louis, MO). bFGF or ^{125}I-bFGF was incubated for 24 h at 35°C with 0.25 M fructose or with 0.25 M glucose-6-phosphate (G6P) in 0.2 M Na_2PO_4, pH 7.8. bFGF or ^{125}I-bFGF was also incubated for 2.5 h with 25 mM glyceraldehyde-3-phosphate (G3P) under identical conditions. Glycosylated bFGFs were divided into aliquots and stored at −70°C.

Determination of extent of nonenzymatic glycosylation. The number of modified amino groups on each of the in vitro glycosylated bFGFs (fructose modified, G6P-modified, and G3P modified) was determined using trinitrobenzenesulfonic acid (TNBS) (Sigma Chemical Co.) according to the method of Steinbrecher (43). Concentration of amino groups was determined by reference to a valine standard. The relative amount of advanced glycosylation endproducts (AGEs) was determined by immunoblotting each of the in vitro modified bFGFs with monoclonal anti-AGE antibody (gift of Drs. Norie Araki and Seikoh Horiuchi, Kumamoto University Medical School, Kumamoto, Japan) as previously described (44). The immunocomplexes were visualized using an ECL kit (Amersham International, Amersham, UK), and intensity was evaluated by scanning densitometry using an Ultrascan XL (LKB Bromma, Sweden).

Heparin–Sepharose affinity chromatography. Equal amounts of ^{125}I-bFGF incubated for 24 h at 35°C in 0.2 M Na_2PO_4 (as a control for the effects of incubation at 35°C), ^{125}I-bFGF mixed with each tested sugar immediately before assay (as a control for effects unrelated to glycosylation), ^{125}I-bFGF glycosylated by fructose, ^{125}I-bFGF glycosylated by G6P, and ^{125}I-bFGF glycosylated by G3P were diluted in 10 mM Tris-HCl/0.1% BSA, pH 7.0, to reduce the NaCl concentration to 0.2 M. The samples were applied directly to 0.6 ml heparin–Sepharose columns (Pharmacia LKB Biotechnology, Piscataway, NJ) equilibrated with 0.2 M NaCl in 10 mM Tris-HCl/0.1% BSA, pH 7.0. The columns were washed with 15 column volumes each of 10 mM Tris-HCl/0.1% BSA, pH 7.0, supplemented with 0.4 M NaCl, 0.9 M NaCl, 1.2 M NaCl, 1.6 M NaCl, and 2.0 M NaCl. The flow rate was 1 ml/min and each wash was collected in one fraction. 0.5 ml of each fraction was counted in a Gamma 5000 gamma counter (Packard Instruments, Meriden, CT). Results were expressed as a percent of the total radioactivity eluted from each column.

Growth assays. Human umbilical vascular endothelial cells from passages 2 to 4 were seeded in 0.2% gelatin-coated 12-well plates (30,000 cells/well) in complete media without human serum and endothelial cell growth factor (ECGF) (negative media). Cultures incubated in negative media with the addition of ECGF or in negative media alone were used as positive and negative controls. Different amounts of bFGF incubated for 24 h with phosphate buffer, bFGF mixed with each tested sugar immediately before assay, and bFGF nonenzymatically glycosylated by either G6P or G3P were added to cultures in negative media. After 5 d the cells were trypsinized and counted using a Coulter Counter. Cell proliferation was expressed as percent of maximal proliferation observed in cells grown in the presence of ECGF (45). bFGF glycosylated by fructose was assayed as described below for determination of intracellular mitogenic activity.

Cell culture conditions. Transformed FBAE GM 7373 cells (46) obtained from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository (Camden, NJ) were maintained in Eagle's minimal essential medium (MEM) containing 10% fetal bovine serum (FBS), vitamins, and essential and nonessential amino acids. To evaluate the effect of high glucose concentration on these cells, confluent GM 7373 cells were incubated for 24 and 168 h in either 5 or 30 mM glucose. These concentrations mimic the levels of extracellular glucose that endothelial cells are exposed to in normal and diabetic subjects, respectively. Culture media was changed daily. The extracellular glucose concentration was measured before each change of media using a Beckman glucose analyzer. Human Umbilical Vein Endothelial Cells (HUVEC) were a gift from Dr. Victor Hatcher (Albert Einstein College of Medicine, Bronx, New York). The cells were grown on 0.2% gelatin-coated 100-mm dishes in M119 medium supplemented with 20% newborn calf serum, 0.5% human serum, 0.08% glutamine, 200 U/ml penicillin, 0.1 mg/100 ml gentamicin, 2.5% heparin, 5% ascorbic acid, and 1% ECGF. NIH 3T3 fibroblasts obtained from the American Type Culture Collection (Rockville, MD) were cultured in DME with 10% calf serum. All medias and all supplements were purchased from GIBCO BRL (Grand Island, NY) except heparin and ascorbic acid, which were obtained from Sigma Chemical Co. ECGF was a gift from Dr. Victor Hatcher.

Glucose transporter identification. Subcellular fractions of GM 7373 cells were prepared according to the method of Bishoff and Lodish (47). Samples of cell membrane fractions were analyzed by 10% SDS polyacrylamide gel electrophoresis followed by Western blotting on nitrocellulose, to determine both the isotype of glucose transporter and the effect of extracellular glucose concentration on transporter protein expression. The membranes were probed with polyclonal anti-GLUT1 or anti-GLUT4 antibodies (gift from Dr. Maureen Charron, Albert Einstein College of Medicine). The immunocomplexes were visualized using an ECL kit (Amersham International, Amersham, UK) and quantitated by scanning densitometry.

Measurement of glucose transport. Confluent GM 7373 cells were washed in PBS with 0.1% BSA and suspended to a final concentration of 4×10^6 cells/ml. A solution containing [2-^{3}H]deoxyglucose (30–60 Ci/mmol; New England Nuclear) was added in various concentrations to individual cell suspensions. After 6 min 0.5-ml aliquots were transferred to Eppendorf tubes which contained 0.5 ml each of 50 mM unlabeled 2-deoxyglucose in PBS. The tubes were centrifuged in a microcentrifuge and the cells were washed once with 1 ml of 50 mM unlabeled glucose, lysed with 0.5 ml of 0.1% Triton X-100, and the radioactivity was measured in a scintillation counter (48).

Measurement of intracellular glucose concentration. Confluent GM 7373 cells were lysed by scraping and by three cycles of freezing and thawing. Aliquots of the lysate (50 μl) were incubated for 30 min at 37°C in a reaction mixture containing 50 mM triethanolamine hydrochloride, pH 9, 1 U/ml glucokinase (Sigma Chemical Co.), 2 mM $MgCl_2$, 40 μM ATP and 30,000 cpm of ^{32}P-ATP (> 4,000 Ci/mmol; New England Nuclear). 1 ml of 1 N perchloric acid containing 0.1 mM phosphoric acid was then added and the samples were incubated at 95°C for 40 min. 200 μl of 100 mM ammonium molybdate and 200 μl of 200 mM triethylamine were added with mixing, and the samples were centrifuged at 3,000 *g* for 20 min. 500-μl aliquots of supernatant were counted in 4.5 ml of Aquasol (DuPont, Boston, MA). This assay is sensitive over a range of 5–3,000 pmoles (49).

Measurement of total intracellular AGE protein. Confluent GM 7373 cells were scraped from flasks and the cytosol fractions were prepared as described previously (47). Different amounts of these fractions were applied to nitrocellulose membranes using a Manifold II Slot Blot System (Schleischer & Schuell, Inc., Keene NH). The membranes were blotted with monoclonal anti-AGE antibody as described previously (44). Band intensity was evaluated by scanning densitometry.

Endothelial cell cytosol preparation. Three confluent 100-mm dishes of GM 7373 cells were wounded by scraping with a rubber policeman in 2 ml of DME/0.1% BSA as previously described (41). The wounded cells were allowed to stand for 20 min at 37°C, after which the suspensions were centrifuged at 2,500 rpm for 5 min at 20°C. The pellets were discarded and the supernatants were assayed for growth-promoting activity.

Assay of intracellular mitogenic activity. Mitogenic activity was measured as incorporation of [^{3}H]thymidine into TCA-insoluble material derived from quiescent cultures of NIH 3T3 fibroblasts incubated with different dilutions of media containing endothelial cell cytoplasm. The background cpm measured from cultures that received DME with 0.1% BSA alone were subtracted. The results are expressed as percentage of maximal cell growth obtained under the same experimental conditions with 0.5 ng of bFGF (50).

Neutralization of mitogenic activity with bFGF antibodies. Different dilutions of media containing endothelial cell cytoplasm were incubated with 266.6 μg/ml of polyclonal anti-bFGF IgG (R&D Systems) for 18 h at 4°C. Samples were assayed for mitogenic activity. A parallel series

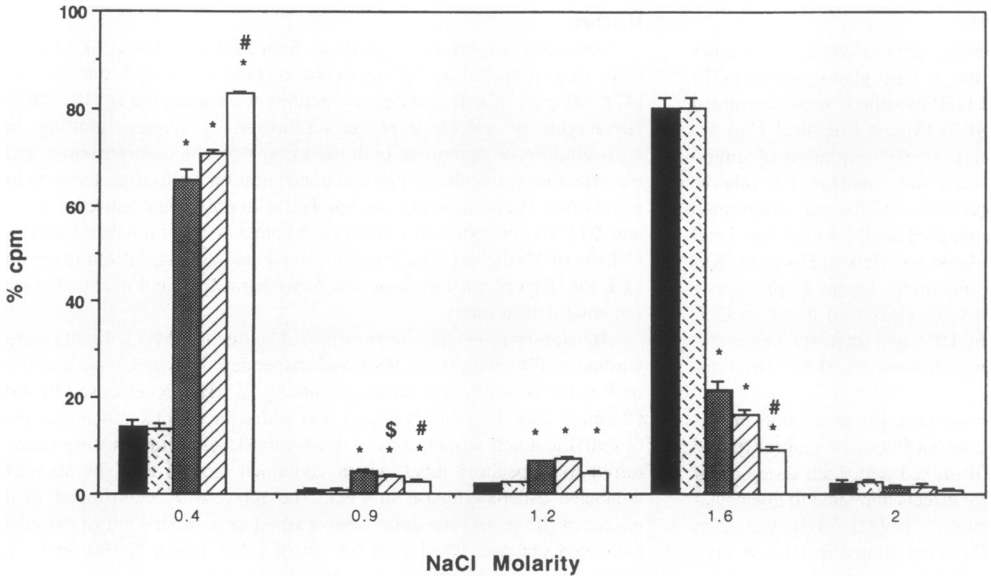

Figure 1. Effect of nonenzymatic glycosylation on bFGF heparin-binding affinity. Equal amounts of unmodified ^{125}I-bFGF incubated for 24 h at 35°C (■), ^{125}I-bFGF mixed with each sugar immediately prior to assay (▨), ^{125}I-bFGF glycosylated with fructose, (■), ^{125}I-bFGF glycosylated with G6P (▨), or ^{125}I-bFGF glycosylated with G3P (□) were applied to 0.6 ml heparin–Sepharose columns and the proteins were eluted with a step-gradient of 0.4–2 M NaCl, as described in Methods. The amount of radioactivity eluted by each salt concentration is expressed as the mean percent ±SE of total CPM eluted. The fraction of total applied radioactivity eluted was 81.6±3.1%. $n = 3$. * $P < 0.05$, each glycosylated bFGF vs controls. # $P < 0.05$ G3P-bFGF vs G6P-bFGF and G3P-bFGF vs fructose-bFGF. $ $P < 0.05$ G6P-bFGF vs fructose-bFGF.

of different dilutions of media incubated with 266.6 μg/ml of BSA for 18 h at 4°C were assayed as controls. The specificity of the antibody used has been previously demonstrated (51).

Western blot analysis. Different amounts of bFGF, bFGF nonenzymatically glycosylated by either fructose, G6P, or G3P and media containing endothelial cell cytoplasm were run in SDS 12% polyacrylamide gels under reducing conditions. The gels were electrophoretically transferred to a nitrocellulose membrane and were blotted with a 1:200 dilution of polyclonal anti-bFGF IgG (R&D Systems) overnight at 4°C. The immunocomplexes were visualized using an ECL kit (Amersham International, Amersham, UK) and quantitated by scanning densitometry.

bFGF ELISA. The bFGF content of the endothelial cell cytosol preparation was determined by using a sandwich ELISA (Quantikine; R&D Systems) according to the manufacturer's instructions.

bFGF AGE determination. Different amounts of media containing endothelial cell cytoplasm were run in SDS 12% polyacrylamide gels under reducing conditions. The gels were electrophoretically transferred to a nitrocellulose membrane and blotted with monoclonal anti-AGE antibody (44). Band intensity was evaluated by scanning densitometry.

Results

Fructose, G6P, and G3P modify different numbers of bFGF lysine residues and form different amounts of AGEs. The number of bFGF lysine residues modified by each sugar was evaluated by the reduction of the amount of bFGF free lysines reacting with TNBS. Incubation with 0.25 M fructose or 0.25 M G6P for 24 h modified 1.3±0.1 and 4.2±0.1 residues/molecule of bFGF, respectively. Incubation with 0.025 M G3P for 2.5 h modified 4.6±0.3 residues/molecule of bFGF. The results are the means±SE of three different experiments. The relative AGE content of each in vitro glycosylated bFGF (fructose modified, G6P modified, and G3P modified) was evaluated by immunoblotting equal amounts of each modified protein with monoclonal AGE antibodies and quantitating by scanning densitometry. After 24 h of incubation with fructose or G6P, the relative content of AGE-bFGF was 0.92±0.08 and 4.1±0.07 AU*mm, respectively. After 2.5 h of incubation with 0.025 M G3P, the AGE content was 6.8±0.03 AU*mm. The results are the means±SE of three different experiments.

In vitro nonenzymatic glycosylation of bFGF reduces its heparin-binding activity. bFGF can bind to its high affinity receptor only when bound to either cell surface heparan sulfate proteoglycans or to free soluble heparin-like molecules. These molecules induce a receptor-compatible conformational change in bFGF that allows the growth factor to bind to its receptor. Because heparin-bFGF interaction is an essential prerequisite for the presentation and subsequent binding of the growth factor to signal transducing receptors, the effect of nonenzymatic glycosylation on bFGF heparin-binding affinity was evaluated using heparin–Sepharose chromatography. As shown in Fig. 1, 80.2% of unmodified bFGF (incubated for 24 h at 35°C) eluted with buffer containing 1.6 M NaCl. Identical results were obtained when bFGF was mixed with each of the sugars tested and immediately applied to the columns. In contrast, after 24 h of incubation with 250 mM fructose, 65.2% of bFGF was eluted with 0.4 M NaCl, and 21.4% with 1.6 M NaCl. Similarly, after 24 h of incubation with G6P, 68.9% of bFGF was eluted with buffer containing 0.4 M NaCl, while only 18.5% eluted with 1.6 M salt. When bFGF was incubated with 25 mM G3P under the same conditions for 2.5 h, 83% of the modified protein was eluted by 0.4 M NaCl and only 9% eluted with 1.6 M salt.

In vitro nonenzymatic glycosylation of bFGF reduces its mitogenic activity. The striking reduction in bFGF heparin-binding activity caused by nonenzymatic glycosylation was closely

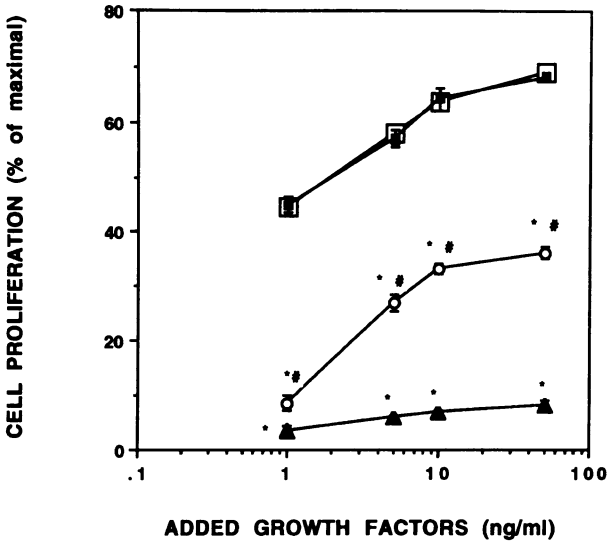

Figure 2. Effect of nonenzymatic glycosylation on bFGF mitogenic activity. Aliquots (1–50 μg) of bFGF incubated 24 h at 35°C (□), bFGF mixed with each sugar immediately before assay (■), bFGF-G6P (○) and bFGF-G3P (▲) were added to HUVEC cells in negative media (media without ECGF). After 5 d the cells were trypsinized and counted. The results are expressed as mean±SE of percent of maximal proliferation obtained in the presence of ECGF. $n = 4$. $^{*}P < 0.05$ modified bFGF vs controls. $^{\#}P < 0.05$ bFGF-G6P vs bFGF-G3P.

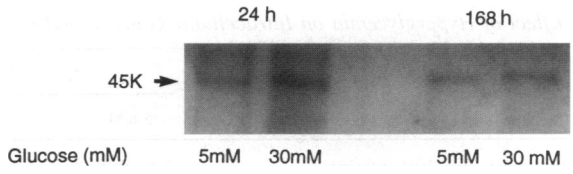

Figure 3. Immunoblot analysis of GLUT-1 transporter in GM7373 cells exposed to different glucose concentrations. Membrane fractions were prepared from confluent cells pre-exposed to 5 or 30 mM glucose for 24 or 168 h. 30 μg of protein were resolved on 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes and blotted with polyclonal anti–GLUT-1 antibody. The immunocomplexes were visualized using horseradish peroxidase-linked anti–rabbit IgG. The density of the bands was evaluated by scanning densitometry and expressed as means±SE of AU*mm. $n = 3$. At 24 h 5 mM = 0.4±0.03 AU*mm, 30 mM = 0.4±0.06 AU*mm. At 168 h 5 mM = 0.41±0.08 AU*mm, and 30 mM = 0.42±0.01. P = NS for all comparisons.

paralleled by a reduction in mitogenic activity (Fig. 2). At most concentrations of protein shown, bFGF glycosylated by G6P had 50% less mitogenic activity than unmodified bFGF incubated for 24 h at 35°C ($P < 0.05$). Similarly, at each protein concentration shown, bFGF glycosylated by G3P had 90% less mitogenic activity than unmodified bFGF incubated for 24 h at 35°C ($P < 0.05$). bFGF mixed with each sugar tested and immediately assayed had identical mitogenic activity to unmodified bFGF. Using NIH 3T3 cells, the mitogenic activity of bFGF glycosylated by fructose was reduced 41% ($P < 0.05$), (data not shown).

GLUT-1 transporters are not downregulated by increased glucose concentration in GM7373 endothelial cells. In cell types not damaged by diabetic hyperglycemia, elevated extracellular glucose concentration has been shown to downregulate glucose transporters (52–54). Therefore, to establish that endothelial-derived GM 7373 cells were an appropriate model in which to investigate the effects of high glucose concentration on intracellular nonenzymatic glycosylation, it was first necessary to establish that glucose transporters were not downregulated by increased glucose concentration. Western blots of GM7373 cells grown in either 5 or 30 mM glucose for 24 and 168 h showed that GLUT1 was the major transporter protein for these cells (Fig. 3). A single band corresponding to a 45-kD protein was detected in the membrane fraction by polyclonal antibodies raised against the purified human erythrocyte transporter, while no band was detected using antibodies to GLUT4. Exposure of GM7373 cells to elevated extracellular glucose did not induce a change in the expression of GLUT1 at either 24 or 168 h. Thus, these cells lack the autoregulatory mechanism for downregulating glucose transport, and would thus be expected to reflect diabetic-like elevations in extracellular glucose concentration in the intracellular compartment.

Increased extracellular glucose concentration accelerates the rate of glucose transport, raises intracellular glucose concentration, and elevates the level of AGE-protein in the cytosol of GM7373 cells. The effects of increased extracellular glucose concentration on glucose transport, intracellular glucose concentration, and the intracellular content of AGE-proteins are shown in Table I. The rate of glucose transport was increased 11.6-fold after exposure to 30 mM glucose for 24 h.

Intracellular glucose concentration increased more than sixfold after exposure to 30 mM glucose for 24 h. After 168 h, intracellular glucose concentration remained nearly five-fold higher than in cells exposed to 5 mM glucose. The amount of AGE-modified protein detected in the cytosol of GM7373 cells was unchanged after 24 h exposure to 30 mM glucose. However, after 168 h AGE-protein in cells exposed to 30 mM glucose was 13.9-fold higher than in cells exposed to 5 mM glucose (Table I).

Conditions producing increased intracellular nonenzymatic glycosylation reduce mitogenic activity of endothelial cell cytosol. Since exposure of GM7373 cells to 30 mM glucose was shown to increase intracellular glucose concentration and intracellular nonenzymatic glycosylation, the effect of these processes on the mitogenic activity of endothelial cell cytosol preparations was evaluated (Fig. 4). When preparations from cells exposed to 30 mM glucose for 168 h were compared with preparations from cells exposed to 5 mM glucose, the capacity to induce DNA synthesis in the NIH 3T3 cell reporter system was decreased ~ 70% at all dilutions tested. This observation suggests that intracellular hyperglycemia and nonenzymatic glycosylation are associated with decreased mitogenic activity, but it doesn't identify the factor(s) responsible.

Anti-bFGF antibody neutralizes the mitogenic activity of endothelial cell cytosol. To determine the relative contribution of bFGF to the mitogenic activity of endothelial cell cytosol, preparations from cells exposed to both 5 and 30 mM glucose were preincubated with polyclonal antiserum to bFGF and then tested for mitogenic activity (Fig. 5). Preincubation with this antiserum completely neutralized the mitogenic activity of the endothelial cell cytosolic preparations from both 5 and 30 mM glucose incubations. Since the anti-bFGF antibody utilized has been shown to be specific for bFGF over aFGF, and to have no effect on cell growth induced by other factors (51), the endothelial cytoplasmic activity inhibited by conditions produc-

*Table I. Effect of Hyperglycemia on Intracellular Glucose and on Glycosylation Product Levels**

	24 h		168 h	
	5 mM	30 mM	5 mM	30 mM
Glucose transport (pmoles/μg/min)	2.6±0.2	29.8±5.1‡	3.2±0.05	24.9±6.7‡
Intracellular glucose (pmoles/μg)	8.2±3.8	55.2±12.9‡	5.1±0.3	26.3±0.5‡
Cytosol AGE-proteins (AU* mm)	0.2±0.03	0.2±0.8	0.18±0.02	2.65±0.12‡

* The results show the mean ± SE of three experiments. ‡ $P < 0.05$ 30 mM vs 5 mM.

ing increased intracellular nonenzymatic glycosylation can be attributed specifically to bFGF.

Nonenzymatically glycosylated bFGF is detected by anti-bFGF antibody. To determine whether polyclonal anti-bFGF serum reacts equally well with nonenzymatically glycosylated bFGF, identical amounts of modified and unmodified protein were immunoblotted using this antiserum and quantitated by scanning densitometry. bFGF glycosylated with either fructose, G6P or G3P was detected at the same intensity as unmodified bFGF from 1 μg to 15 pg. Modified and unmodified bFGFs all became undetectable at the same subdilution (data not shown).

Conditions producing increased intracellular nonenzymatic glycosylation do not decrease bFGF protein expression. Having established that nonenzymatic glycosylation of bFGF did not interfere with its immunodetection, the effect of conditions producing increased intracellular nonenzymatic glycosylation on the level of immunodetectable bFGF was evaluated in endothelial cell cytosolic preparations from both 5 and 30 mM glucose incubations. If the previously observed reduction in mitogenic activity reflected a reduction in bFGF expression and protein concentration, a decrease of bFGF would be expected on the immunoblot from the cells exposed to 30 mM glucose. However, the single 18-kD bFGF band present in each of the samples analyzed was of the same intensity (5 mM glucose cells 0.5±0.04 AU*mm, 30 mM glucose cells 0.5±0.03 AU*mm). Direct determination of bFGF content by ELISA showed equivalent amounts in both samples (5 mM glucose cells 4.48±0.18 ng/10.5^6 cells, 30 mM glucose cells 4.57±0.12 ng/10.5^6 cells). The findings of no decrease in bFGF protein together with a marked decrease in bFGF mitogenic activity are consistent with a posttranslational modification of bFGF such as nonenzymatic glycosylation.

Nonenzymatically glycosylated bFGF in the cytosol extract is detected by anti-AGE antibody. Cytosolic preparations from both 5 and 30 mM glucose incubations were immunoblotted with anti-AGE antibody and quantitated by scanning densitometry. Parallel lanes of the same gel were immunoblotted with anti-bFGF antibody to indicate the location of intracellular bFGF (Fig. 6). The 18-kD band corresponding to bFGF in the 30-mM glucose preparation showed a 6.1-fold increase in AGE-content compared to the 5-mM preparations (30 mM glucose cytosol 0.80±0.09 AU*mm, 5 mM glucose cells 0.13±0.06 AU*mm).

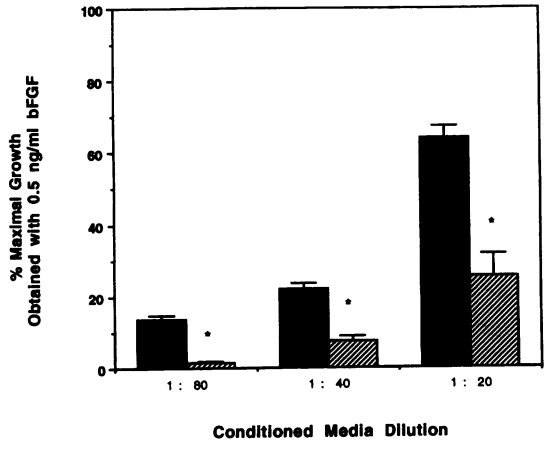

Figure 4. Mitogenic activity of GM7373 endothelial cell cytosol after exposure to different extracellular glucose concentrations. Indicated dilutions of cytosol preparations obtained by scraping confluent GM7373 cells pre-exposed to 5 (■) or 30 mM (■) glucose for 168 h were added to quiescent cultures of NIH 3T3 cells with 1 μC of [^{3}H]thymidine. After 24 h, the amount of radioactivity incorporated into trichloroacetic acid-precipitable material was measured. The background CPM measured from cultures that received DME 0.1% BSA alone were subtracted. The results are expressed as means±SE of percent of the maximal cell growth obtained with 0.5 ng bFGF. n = 3. *$P < 0.05$ 30 vs 5 mM at each dilution tested.

Discussion

The results of this study demonstrate that nonenzymatic glycosylation of bFGF with the intracellular sugars fructose, G6P

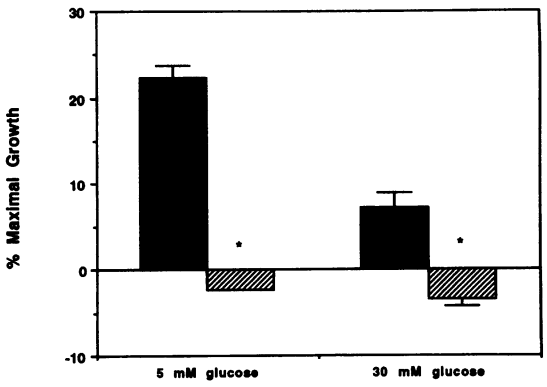

Figure 5. Neutralization of GM7373 endothelial cell cytosol mitogenic activity by polyclonal anti-bFGF antibody. Cytosolic preparations from confluent GM7373 cells pre-exposed to 5 or 30 mM glucose for 168 h. were incubated with the BSA alone (■) or a polyclonal anti-bFGF IgG (■) for 18 h at 4°C. Mitogenic activity was then assessed using a standard 3T3 cell assay as described in Methods. Results are expressed as means±SE of percent of cell growth obtained with 0.5 ng of bFGF. $n = 3$. *$P < 0.05$ + IgG vs −IgG.

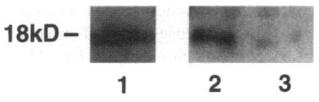

Figure 6. Immunoblot of AGE-bFGF from GM7373 endothelial cell cytosol after cell culture in different glucose concentrations. 100 pg of recombinant bFGF (lane *1*) or 50 μg of cytosolic preparations obtained from confluent GM7373 cells pre-exposed toeither 5 mM glucose (lane *3*) or 30 mM glucose (lane *2*) for 168 h were resolved on 12% SDS polyacrylamide gels, transferred to a nitrocellulose membrane and blotted with either a polyclonal anti-bFGF antibody (lane *1*) or with an anti-AGE antibody. The immunocomplexes were visualized by using horseradish peroxidase-linked anti–rabbit IgG. The density of the bands was evaluated by scanning densitometry and expressed as means±SE of AU*mm. n = 3. 5 mM = 0.13±0.06 AU*mm, 30 mM = 0.8±0.09 AU*mm. $P < 0.05$, 5 vs 30 mM.

and G3P markedly reduces both its high affinity heparin-binding capacity and its mitogenic activity. In GM7373 endothelial cells, bFGF-dependent mitogenic activity is greatly reduced by raising the extracellular glucose concentration, while the rate of glucose transport, intracellular glucose concentration, and total nonenzymatically glycosylated protein in the cytosol are all increased. The amount of detectable bFGF protein is not changed, however, suggesting that the observed decrease in bFGF mitogenic activity is caused by a post-translational modification of bFGF induced by elevated glucose concentration. Relative AGE-bFGF content in cells exposed to high glucose is increased 6.1-fold. These data are consistent with the hypothesis that nonenzymatic glycosylation of intracellular protein alters vascular cell function, since bFGF plays an important role in regulating normal endothelial cell growth and basement membrane production (55–62).

The heparin-binding domain of bFGF has been shown previously to reside in the carboxylterminus of the molecule from Asn^{101} to Pro^{141}, although truncation of even seven amino acid residues from the carboxyl-terminus (including Lys^{145}) significantly reduces bFGF's affinity for heparin (63). In contrast, the mitogenic receptor binding activity of bFGF appears to be associated with two other regions of the molecule, amino acid residues 33–77 and 102–129 (64). Modification of 1.3 lysine residues per molecule of bFGF by fructose caused a loss of heparin-binding affinity similar to modification of over four residues per molecule by both G6P and G3P, suggesting that heparin-binding affinity is primarily dependent on one critical residue in the heparin binding region. This loss of bFGF heparin-binding affinity by itself appears sufficient to reduce its mitogenic activity by 40–50%. The further two-fold loss of mitogenic activity associated with glycosylation by G3P compared to glycosylation by G6P or fructose most likely results from differences in the type of glycosylation (early vs. advanced products), since the relative AGE-bFGF content generated by G3P was 7.4-fold higher than that generated by G6P, while the total number of lysine residues modified by these two sugars was the same. AGEs in the mitogenic receptor binding region may critically impair function where early glycosylation products do not.

To investigate the effects of bFGF glycosylation in GM7373 endothelial cells, we first demonstrated that GLUT-1 transporters are not downregulated by increased glucose concentration at either 24 or 168 h. This observation is consistent with that previously reported for bovine aortic endothelial cells incubated for 24 h in either 5.5 or 22 mM glucose (52). In that report, bovine aortic smooth muscle cells decreased the level of GLUT-1 protein when medium glucose was increased, consistent with a compensatory decrease in glucose transport that protects non-endothelial cells from intracellular hyperglycemia.

Consistent with the finding that increased extracellular glucose concentration does not downregulate GLUT-1 transporters in GM7373 endothelial cells, it increases the rate of glucose transport, elevates intracellular glucose concentration, and raises the level of total AGE-protein in the cytosol. Although the magnitude of the increase in mean intracellular glucose concentration induced by 30 mM glucose declines from sixfold to fivefold from 24 h to 168 h, the difference is not statistically significant. However, the differences between glucose transport and intracellular glucose concentration at 30 and 5 mM glucose are statistically significant ($P < .05$) at both 24 and 168 h.

At 24 h, the level of cytosol AGE-proteins was equal at both glucose concentrations. By 168 h, the level after exposure to 30 mM glucose was 13.9-fold higher than after exposure to 5 mM glucose. Since 7 d is not sufficient to reach a steady state even of early protein glycosylation products with glucose, the unexpected magnitude of this increase of AGE-proteins in cells exposed to 30 mM glucose most likely results from glycosylation by more highly reactive intracellular sugars and glucosones (65).

At the same time that increased extracellular glucose concentration increases the rate of glucose transport, elevates intracellular glucose concentration, and raises the level of total AGE-protein and AGE-bFGF in the cytosol of GM7373 endothelial cells, it dramatically decreases the level of mitogenic activity. All of the mitogenic activity in the cytosol of cells grown in either 5 or 30 mM glucose was neutralized by a polyclonal anti-bFGF antibody, showing that the decreased mitogenic activity induced by incubation of cells in 30 mM glucose was mediated by a specific effect on bFGF. This effect did not appear to involve altered bFGF production, since immunoblotting and ELISA assay showed that 30 mM glucose did not decrease detectable bFGF protein. However, relative AGE-bFGF content was increased 6.1-fold in cytosol from these cells. Together, these data strongly support the hypothesis that elevated extracellular glucose concentration increases nonenzymatic glycosylation of bFGF, thereby reducing its mitogenic activity.

Hyperglycemia-induced decreases in bFGF mitogenic activity could explain several important features of diabetic vascular disease. In diabetic retinopathy, loss of capillary endothelial cells is a characteristic finding. In the post-infarction diabetic myocardium, there is a decrease of capillary density consistent with a subnormal response to the stimulus of ischemia (66). A variety of other intracellular proteins likely undergo nonenzymatic glycosylation when the extracellular glucose concentration is elevated. The identification of additional specific targets for intracellular glycosylation and the elucidation of the functional consequences of this process should provide further insight into the pathogenesis of diabetic vascular disease.

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